

modulate their adhesive and motility activities through a poorly understood mechanism involving Rho-family GTPases. Additionally, p120, and likely ARVCF, interacts with transcription factors in the nucleus to regulate gene expression in analogy to the well-characterized β -catenin. To better understand the role of catenins, we employed yeast two-hybrid analysis using *Xenopus* ARVCF as the 'bait' and successfully isolated three novel interacting proteins of distinct functional classes. Initially termed ARVCF-associated proteins (AAPs), the first, AAP1, shares a loose similarity to actin-remodeling proteins and was found to contain an ATPase site. AAP2 is homologous to a RNA-binding protein of unknown function, and AAP3 shares homology with several signal transduction molecules. All three proteins have proven to interact with ARVCF not only in the yeast two-hybrid system, but also to interact with ARVCF in vitro using a coupled transcription and translation system followed by co-immunoprecipitation (co-IP). To further confirm these interactions, the three putative interactors were co-expressed with ARVCF or p120 in *Xenopus* embryos and association assayed by co-IP. Surprisingly, AAP1 was found to specifically interact with ARVCF, and did not associate with p120, suggesting a unique role for ARVCF at the adherens junction and its possible contribution to the modulation of the actin-cadherin interaction. AAP2 and 3 both interacted with ARVCF and p120, indicating they may represent a broader class of p120-family member associating factors. Further study of these interactions will shed light on the function of ARVCF and p120 and their roles at the adherens junction.

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Role of G protein-coupled Receptor Induced Cell-cell Junctions in Vascular Maturation

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The integrity of the vascular system depends on the proper interactions between vascular endothelial cells (EC) and pericytes. Abnormal pericyte coverage of the microvessels leads to vascular dysfunction and severe tissue damage, as exemplified in diabetic retinopathy. Indeed, normal vascular development requires the recruitment of pericytes to nascent vessels, a process known as vascular maturation. Genes such as LKLF transcription factor, platelet-derived growth factor (PDGF)- β receptor, angiopoietin, TGF- β receptor endoglin and the sphingosine 1-phosphate (S1P) receptor S1P1- (also known as EDG-1) are essential for vascular maturation; however, the molecular basis of this important process remains a mystery. We provide a mechanistic basis for vascular maturation, whereby, interaction of the bioactive lipid S1P with its G-protein-coupled receptor (GPCR) S1P1 induces the assembly of N-cadherin-based heterotypic cell-cell junctions between EC and pericytes. Regulated formation of heterotypic cell-cell junctions between vascular cells and mural cells may be an important aspect of vascular development and function.

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Mechanotransduction by VE-Cadherin Controls Endothelial Cell Proliferation

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Cadherins are known to transduce chemical cues and thereby influence cell proliferation through several signaling pathways. However, the ability of cadherin-based adhesions to affect cell growth through the transduction of mechanical signals has not yet been examined. Our laboratory has previously demonstrated that changes in cell spreading can regulate endothelial cell proliferation by altering cytoskeletal mechanics. By independently controlling cell-cell contact and cell spreading, we have subsequently shown that the engagement of cell-cell contact through VE-cadherin generates opposing signals for proliferation. VE-cadherin inhibited growth by decreasing cell spreading, while simultaneously promoted growth via a spreading-independent mechanism. The observed proliferation rate of the cell population depended on how the adhesive microenvironment affected cell spreading, and hence the balance of these two cues. The newly found cadherin-induced stimulatory signal was dependent on both mediators of growth factor signaling and actin cytoskeletal organization. We now present evidence to suggest that cadherin engagement acts as a mechanical signal by increasing intracellular tension and altering the localization of several focal adhesion proteins. These data highlight the importance of crosstalk through cell-cell contact, cell-ECM adhesion, and cytoskeletal structure in influencing cell fate decisions.

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Differential Involvement of β 3 Integrins in Transendothelial Migration of Human Prostate Cancer Cell Lines

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β 3 integrins play a role in metastatic progression of prostate cancer by mediating adhesion of cancer cells to endothelial cells and their migration through

extracellular matrix (ECM). However, the involvement of β 3 integrins during transendothelial migration (TEM) of prostate cancer cells is unclear. In this study, we used an *in vitro* assay to determine the role of β 3 integrins in TEM of 3 human prostate cancer cell lines through a monolayer of human lung microvascular endothelial cells (HMVEC-L). Western blot and immunocytochemistry analyses demonstrated that the expression of β 3 integrins was higher in DU145 cells than in PC3 cells. Whereas in both cell types β 3 integrins were located on the cell surface, in PC3 cells they were, in addition, located in the cytoplasm but failed to cluster in focal contacts with the ECM. After 3 hours of coculture with HMVEC-L, 78% of adherent DU145 cells and 50% of adherent PC3 cells initiated TEM. In contrast, LNCaP cells expressed little β 3 integrins and only 15% of cells initiated TEM. Blockade with a β 3 integrin monoclonal antibody inhibited the number of migrating PC3 cells from 50% to 26% and most migrating cells were blocked at an early migratory stage as assessed by F-actin labelling. The antibody blockade, however, did not affect TEM of DU145 cells. Our data revealed that the expression of β 3 integrins was tightly correlated with the migratory behaviour of the prostate cancer cell lines examined, and suggest that β 3 integrins play important roles during TEM of PC3 cells. This work was supported by the Cancer Research Society of Canada.

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Upregulation of VCAM (CD106) by 5-Hydroxytryptamine (Serotonin) in Human Umbilical Vein Endothelial Cells

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Endothelial cells are integrally involved in the inflammatory process by providing a surface for leukocyte attachment. The mechanisms for leukocyte adhesion and extravasation involve a number of inflammatory cytokines and a variety of adhesion molecules and their ligands. Initially, neutrophils bind loosely to selectins, then tumble downstream along the endothelial cell, ultimately binding tightly to ICAM, VCAM, and PECAM. Although research supports upregulation of VCAM (CD106) by tumor necrosis factor (TNF), little is known about the role of 5-hydroxytryptamine (5-HT, serotonin) in the upregulation of this adhesion molecule. Released from aggregating platelets, 5-HT is present during inflammation, causing increased vascular permeability and smooth muscle contraction in local arterioles and venules. This study investigated whether 5-HT has a role in VCAM upregulation. Human umbilical vein endothelial cells (HUVECs) were cultured and incubated for 12 and 24 hours with 1 mM 5-HT. Fixed and permeabilized with formaldehyde and Triton-X, immunofluorescence labeling studies indicated that VCAM was present in HUVECs treated with 5-HT. Cells incubated with TNF, a cytokine known to upregulate VCAM, was used as a positive control and showed abundant cytoplasmic and cell membrane bound VCAM. Compared to the TNF-treated cells, the HUVECs treated with 5-HT indicated less VCAM. However, the 5-HT treated cells demonstrated a punctate pattern on the cell membrane and increased intracellular fluorescence not seen in untreated HUVECs controls. These findings suggest that 5-HT may have a role in VCAM upregulation, thus providing an additional stimulus to enhance neutrophil binding and extravasation during an inflammatory response.

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Human Breast Cancer Cells Use β 1 Integrins to Selectively Adhere to and Migrate Across Lung Microvessel Endothelial Cells

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Breast tumors preferentially metastasize to lung, though the basis for this selectivity is not well understood. We have developed an *in vitro* model of tumor cell extravasation, using Electric Cell-substrate Impedance Sensing (ECIS)-based measurements of endothelial cell monolayer integrity. Confluent endothelial cell layers are challenged with breast tumor cells or their non-tumorigenic counterparts and impedance changes across the monolayer are measured in real time. Successful transendothelial migration is characterized by a sharp drop in impedance, reflecting a rupturing of the junctional complexes between endothelial cells. Using this system, we have observed that human AU-565 breast cancer cells adhere to pulmonary artery and lung microvessel endothelial cells but migrate preferentially across the microvessel endothelial cells. This migration occurs in serum-free, defined medium lacking soluble growth factors, suggesting that endothelial cell activation is not necessary to allow tumor cell extravasation. MCF-10A cells, which are non-tumorigenic human breast epithelial cells, adhere weakly to but do not penetrate monolayers of either type of endothelial cells. Adhesion and transendothelial migration of AU-565 cells is inhibited by β 1 integrin function blocking antibodies, demonstrating that β 1 integrins play a key role in mediating the selective extravasation of breast tumor cells into lung.

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VCIP Induces Cell-Cell Interactions: Its Role in Angiogenesis

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Angiogenesis is not only required for normal physiology, but is also vital for many diseases that depend upon growth of blood vessels including growth of solid tumors, diabetic retinopathy, and cardiovascular diseases. Endothelial cells (ECs) that line the blood vessels play direct role in the formation of new blood

vessels. Upon activation ECs elongate extensively and form cell-cell interactions, the molecules and mechanisms that control these events are not entirely understood. By employing subtractive suppression hybridization and differential display technique, we identified and cloned an induced gene from ECs undergoing capillary morphogenesis that we named as VCIP for VEGF & type I Collagen Inducible Protein. Herein, we demonstrate that endogenous and recombinant VCIP proteins are expressed as N-glycosylated and non-glycosylated forms of ~38 and 46/48 kDa molecular masses. Immunofluorescent localization and cell surface biotinylation followed by immunoprecipitation assay show that VCIP is a cell surface protein. Encouraged by its atypical membrane anchoring structure we hypothesized that VCIP can induce and organize both homotypic and heterotypic cell-cell interactions. In support of this hypothesis, the overexpression of wild-type but not mutant-VCIP promoted cell-cell interactions. In addition, we found that recombinantly expressed VCIP interacted productively with a subset of integrins on ECs; this data was further supported by solid-phase ELISA assay. Interestingly, immunoprecipitation and Western blot analyses suggested that VCIP collaborates with signaling molecules to activate intracellular signaling machinery that includes tyrosine phosphorylation of Fak and Shc, molecules required for EC migration and differentiation. Importantly, immunostaining data showed that VCIP was strongly coexpressed with VEGF, MMP2, and avb3 integrin in tumor vasculature including angioma, hemangioma, and melanoma. Based upon our collective results we propose that VCIP nucleates a unique cell-cell interaction, a process necessary for normal and pathological angiogenesis.

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Effects of a Monoclonal Antibody to the Eph A2 Receptor on the In Vitro Behavior of Human Microvascular Endothelial Cells

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Eph receptor tyrosine kinases and their ligands, termed ephrins, have been implicated in regulation of cell migration, axonal guidance and vascular network assembly during development. To study the role of Eph family members in endothelial cell behavior, we identified Eph and ephrin family members expressed in Human Microvascular Endothelial Cells from Lung (HMVEC-L) using Flow Cytometry. Expression of EphA2 and its ligand ephrin A1 are particularly prominent in these cells. Treatment of HMVEC-L with a monoclonal antibody directed against the extracellular domain of EphA2 dramatically inhibits tube formation in both a matrigel assay and a co-culture assay with human primary fibroblasts. HMVEC-L migration is also blocked by this Mab in both wound healing and Boyden chamber assays. The mechanism by which this antibody mediates these effects will be further discussed.

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Human T-Cell Lymphotropic Virus Type I Transformed Cells Induce Angiogenesis and Establish Functional Gap Junctions with Endothelial Cells

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Angiogenesis plays a critical role in the growth and metastasis of solid tumors. However, the role of angiogenesis in hematological malignancies was only recently appreciated. Human T cell lymphotropic virus type I (HTLV-I) is the causative agent of adult T-cell leukemia/lymphoma (ATL) and HTLV-I associated myelopathy (TSP/HAM). We show that HTLV-I transformed T-cells, but not HTLV-I negative CD4+ T-cells, secrete biologically active forms of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and, accordingly, induce angiogenesis in vitro. Furthermore, fresh ATL leukemic cells derived from acute ATL patients produce VEGF and bFGF transcripts and proteins. Consequently, ATL and TSP/HAM patients have very high plasma levels of VEGF and bFGF compared to sero-negative controls. As a result, plasmas from ATL and TSP/HAM patients induce angiogenesis in vitro, a phenomenon inhibited by anti-VEGF antibodies. The viral transactivator Tax activates the VEGF promoter, linking induction of angiogenesis to viral gene expression. Angiogenesis is associated with the adhesion of HTLV-I transformed cells to endothelial cells and gap junction-mediated hetero-cellular communication between the two cell types. Angiogenesis induced by HTLV-I infected cells may facilitate central nervous system or visceral invasion. Moreover, since ATL cells express VEGF receptors, such high VEGF concentration may create an autocrine loop that could represent a therapeutic target.

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Sustained Activation of MAPK/ERKs Disrupts Cadherin-Mediated Cell-Cell Adhesion in Endothelial Cells

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Cadherin-mediated cell-cell interactions in endothelial cells are essential for vascular integrity and modulate vascular permeability. We have demonstrated that PECAM-1 isoforms can differentially modulate cadherin-mediated cell-cell

interactions in epithelial cells through sustained activation of MAPK/ERKs. To determine whether sustained activation of MAPK/ERKs is sufficient to disrupt cadherin-mediated cell adhesions in endothelial cells, we transfected mouse brain endothelial (bEND) cells with a dominantly active MEK-1 cDNA (bEND/MEK). bEND/MEK cells exhibited decreased expression of VE-cadherin and catenins concomitant with enhanced ability to migrate and form capillary-like structures on Matrigel. Enhanced migration of these cells was confirmed by monolayer wounding assay. HUVEC incubated with phorbol myristate acetate (PMA) also exhibited sustained activation of MAPK/ERKs and altered cadherin-mediated cell adhesion. This may contribute to PMA mediated enhanced ability of HUVEC to form capillary networks on Matrigel. Inhibition of MAPK/ERKs was sufficient to re-establish VE-cadherin/catenins expression and cell-cell interactions. These results suggest that sustained activation of MAPK/ERKs by specific PECAM-1 isoform(s) can disrupt cadherin-mediated cell adhesions during early phases of angiogenesis facilitating migration. (Supported by NIH grant AR-45599)

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Cortactin—A Major Phosphotyrosin Protein Modulated by Cell-Cell Interaction in Corneal Endothelial Cells

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Bovine corneal endothelial (BCE) cells upon reaching confluence form a full contact inhibited cell monolayer, firmly attached to both extracellular matrix via integrins and to each other by tight and gap junctions. The integrins and cell junctions interact with the cellular cytoskeleton involving various phosphotyrosine proteins. In this work we studied the involvement of cortactin (a phosphotyrosine protein appearing in two bands of 80 and 85 kDa) in these sites in BCE cells. The location of proteins was determined by immunocytochemistry. Specific protein levels and tyrosine phosphorylation levels were determined by immunoradios assays followed by quantitation using image analyzer. The major phosphotyrosine proteins appearing in confluent BCE cells are FAK, vinculin and cortactin (p80/85). In confluent BCE cells cortactin is co-localized with α -catenin at cell-cell contacts, while in migrating cells cortactin is co-localized with FAK at focal contacts. Cortactin and FAK protein levels are significantly decreased by 70-80% upon cell detachment, but not their phosphorylation levels. Cells reattachment is associated with a gradual recovery of the protein level and significant transient increase of 2.5-, 4.5- and 2.5-fold compared to baseline in the phosphorylation level of cortactin p80, cortactin p85 and FAK, respectively. However, vinculin's protein content was slightly declined during detachment (40%) while its phosphorylation level showed a significant decrease of 90%. Both protein and phosphorylation levels of vinculin recovered through the reattachment process. The addition of vanadate (a general tyrosine phosphatase inhibitor) reveals a significant turnover of tyrosine phosphorylation in confluent and during cell reattachment but not in rounding cells. Cortactin (p80/85) is a major phosphotyrosine protein in BCE cells, localized at cell-cell contacts in a confluent cell monolayer. Cortactin protein level and phosphorylation on tyrosine residues is involved in cell-cell interaction.

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Monocytes in Culture Can Cross Endothelial Monolayers Without Disrupting Cell-Cell Junctions

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The migration of monocytes across the vascular endothelium (diapedesis) can occur through endothelial cell-cell junctions (paracellular) or through individual endothelial cells (EC) without the disruption of junctions (transcellular). Transcellular diapedesis has so far only been observed *in vivo* using electron microscopy. *In vitro* studies have provided considerable insight into the mechanisms controlling paracellular diapedesis, however no *in vitro* data exist on the conditions promoting transcellular diapedesis. To address this issue, we added human peripheral blood monocytes (PBM) to monolayers of human coronary EC grown on matrigel and examined diapedesis using confocal microscopy after labelling for endothelial cell-cell junctions and F-actin. We found that under control conditions 18% of migrating monocytes used a transcellular route, without causing EC damage, as judged by F-actin labelling. MCP-1 was able to increase this value by 2-fold, to 35%. We attempted to promote transcellular diapedesis by blocking the paracellular pathway using the src kinase inhibitor PP2, and astrocyte-conditioned media (ACM) to stabilize adherence and tight junctions. Both treatments, when used independently, had no effect on transcellular diapedesis. However, when ACM was used in conjunction with vascular endothelial growth factor (VEGF), an inducer of endothelial caveolae and transcellular vesiculo vacuolar organelles (VVOS), transcellular diapedesis was dramatically upregulated by 4-fold to 61%. Transcellular diapedesis was maximal when the endothelial monolayer was pretreated with 20ng/ml VEGF for 30 minutes prior to monocyte addition. Although the precise conditions and molecular mechanisms are still unclear, our data support the involvement of endothelial caveolae and/or VVOS during transcellular diapedesis. Our *in vitro* model will allow us to further characterize the molecular pathways by which leukocytes cross the endothelium via a transcellular route. Supported by the Heart and Stroke Foundation of Ontario.